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Note

Inactivation of plant and animal viruses by proanthocyanidins from *Alpinia zerumbet* extract

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Abstract *Alpinia zerumbet* (Pers.) B.L. Burtt and R.M. Smith belongs to the *Alpinia* genus in the *Zingiberaceae* family. In East Asia, *Alpinia zerumbet* has been widely used as food and traditional medicine. Previously, we identified proanthocyanidins (PACs), an anti-plant-virus molecule in *A. zerumbet*, using *Nicotiana benthamiana* and tomato mosaic virus (ToMV). Here, we found that PACs from *A. zerumbet*, apple, and green tea effectively inhibited ToMV infection. Additionally, the PACs from *A. zerumbet* exhibited greater antiviral activity than those from apple and green tea. The PACs from *A. zerumbet* also effectively inactivated influenza A virus and porcine epidemic diarrhea virus (PEDV), which acts as a surrogate for human coronaviruses, in a dose-dependent manner. The results from the cytopathic effect assays indicated that 0.1 mg/ml PACs from *A. zerumbet* decreased the titer of influenza A virus and PEDV by >3 log. These findings suggested that the direct treatment of viruses with PACs from *A. zerumbet* before inoculation reduced viral activity; thus, PACs might inhibit infections by an influenza virus, coronaviruses, and plant viruses.

Key words: *Alpinia zerumbet*, antiviral activity, coronavirus, influenza virus, tomato mosaic virus.

Proanthocyanidins (PACs), also known as condensed tannins, are oligomers and polymers of monomeric flavan-3-ols, such as (+)-catechin and (−)-epicatechin, linked through a specific single (B linkage) and double (A linkage) bonds produced by the flavonoid biosynthetic pathway (Beecher 2004; Dixon et al. 2005). The PACs, abundant in the fruits, bark, seeds, flowers, leaves, and nuts of many plants, exhibit antioxidant activity (Koga et al. 1999) and protect plants from microbial pathogens, insect pests, and herbivores (Barbehenn and Constabel 2011; Bernays 1978; de Colmenares et al. 1998; Scalbert 1991).

Previously, we found that *Alpinia zerumbet* extracts contained proanthocyanidins (AzPACs) with high antiviral activity against tomato mosaic virus (ToMV) infection of *Nicotiana benthamiana* of the *Solanaceae* family (Hatanaka et al. 2021; Narusaka et al. 2020). *Alpinia zerumbet* (Pers.) B.L. Burtt and R.M. Smith, commonly known as shell ginger, is a member of the *Alpinia* genus in the *Zingiberaceae* family (Teschke and Xuan 2018). Native to subtropical and tropical regions in East Asia, including the Nansei islands surrounded by the Pacific Ocean and the East China Sea in Japan, *A. zerumbet* is also known as an aromatic and medicinal herb plant. *A. zerumbet* leaves and extracts are used to produce essential oils, herbal tea, flavor noodles, cosmetics, and traditional folk medicine for their anti-inflammatory, antioxidant, bacteriostatic, and fungistatic properties (Elzaawely et al. 2007a, b; Tu and Tawata 2015; Yonaha et al. 2013; Zoghbi et al. 1999). AzPACs are abundant in *A. zerumbet* extracts. Additionally, the AzPACs, comprising mostly epicatechin units linked through a single B-type linkage, exhibit more than 40 degrees of polymerization. However, AzPACs have not been characterized; thus, this study examined their ability to inhibit plant and animal viruses.

The antiviral assay (Narusaka et al. 2020) was performed using the AzPACs, and PACs were purified from apple juice (ApPAC) and green tea (GtPAC) (Hatanaka et al. 2021), which had approximate molecular weights of 14,700, 8,400, and 1,200–1,900, respectively (Supplementary Figure S1). Our results showed that treatments with the PACs effectively protected *N. benthamiana* leaves against ToMV-GFP infection compared with the control (Figure 1). Additionally, AzPAC demonstrated greater antiviral activity than

Abbreviations: ApPAC, apple juice proanthocyanidins; AzPACs, *Alpinia zerumbet* extracts contained proanthocyanidins; CPE, cytopathic effect; DMF, dimethylformamide; GFP, green fluorescent protein; GtPAC, green tea proanthocyanidins; PAC, proanthocyanidin; PEDV, porcine epidemic diarrhea virus; ToMV, tomato mosaic virus.

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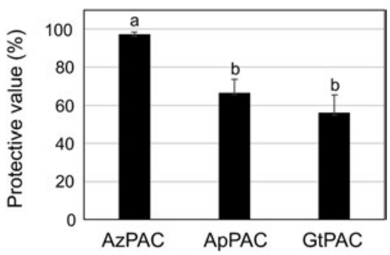

Figure 1. Effects of foliar application of proanthocyanidins on GFP-tagged tomato mosaic virus (ToMV) virions. *Nicotiana benthamiana* plants were treated with water and 1,000 ppm of proanthocyanidins from *Alpinia zerumbet* (AzPAC), apple (ApPAC), and green tea (GtPAC) and then inoculated with GFP-tagged ToMV 3 days after treatment. The number of GFP spots formed on the inoculated leaves was counted at 3 days post-inoculation, and the protected value of each application was calculated. Bars indicate the standard error (SE). The experiment was independently performed twice ($n>3$ per experiment). The differences between the control and treated plants were statistically significant ($p<0.01$). Different letters represent a statistically significant difference ($p<0.05$).

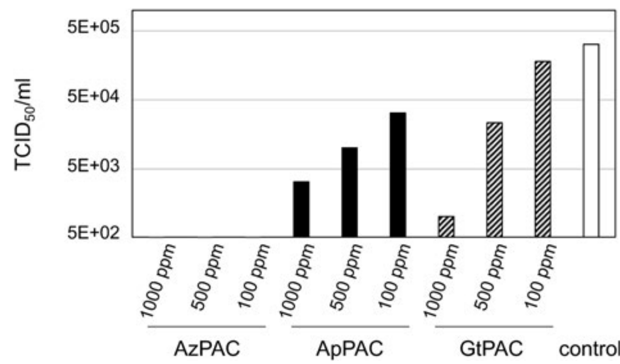

Figure 2. Inactivation of influenza A virus by different concentrations of proanthocyanidins (PACs) from *Alpinia zerumbet*, apple, and green tea after 30 min. PACs were mixed with or without influenza A H1N1 virus (A/PR/8/34) and added to MDCK cells. After incubation for 4–7 days, a cytopathic effect was observed under an inverted microscope. The 50% endpoint dilution ($TCID_{50}$/ml) was calculated using the Reed and Muench (1938) method.

Table 1. Inactivation of porcine epidemic diarrhea virus (PEDV) by different concentrations of proanthocyanidins from *Alpinia zerumbet* after 30 min.

Concentrations of proanthocyanidin (ppm)	Contact time (min)	
	0	30
0 (control)	$10^{6.5}$*	$10^{6.5}$
100		$<10^{2.5}$
500		$<10^{2.5}$

* The 50% endpoint dilution ($TCID_{50}$/ml) was calculated by the Reed and Muench (1938) method.

GtPAC and ApPAC.

During the development of antiviral drugs, medicinal plants have been explored as alternative sources of agents against human and animal viruses (Kwon et al. 2010; Sawamura et al. 2010; Shoji et al. 2017; Watanabe et al. 2011). For example, the influenza virus and coronaviruses are enveloped RNA viruses that can cause outbreaks and even pandemics; particularly, the H1N1 influenza virus and the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have caused pandemics (Nicholson et al. 2003; Wölfel et al. 2020). Here, we examined whether the influenza A virus and porcine epidemic diarrhea virus (PEDV), which acts as a surrogate for human coronaviruses, could be inactivated by exposure to PACs.

The effect of PACs on influenza and PEDV infections, which was an indicator of the antiviral activity of the PACs, was investigated using a cytopathic effect (CPE) assay. The treatments with the PACs dose-dependently inhibited CPE in the cells infected with influenza A virus compared with those in the control (Figure 2; Supplementary Figure S2). Additionally, AzPAC showed greater antiviral activity against influenza A virus than GtPAC and ApPAC; 0.1 mg/ml AzPACs decreased the titer of influenza A virus by >3 logs. Then, the CPE assay with PEDV was used to examine whether AzPACs could prevent PEDV infection (Table 1). AzPACs remarkably inhibited CPE compared with the control; AzPACs at 0.1 mg/ml decreased the PEDV titer by >4 logs.

In conclusion, PACs, especially AzPACs, effectively inactivated influenza A virus and PEDV in vitro in a dose-dependent manner along with inhibiting ToMV infection. The findings suggested AzPACs had broad-spectrum antiviral activities against various viruses. The PACs are polyphenolic compounds in *A. zerumbet* extracts and likely contribute to most of their biological effects. PACs also have beneficial effects on human health and activities against infectious diseases. PAC-containing plant materials have been used as food, and PACs are safer than other chemicals. Thus, AzPAC is a potential lead compound for developing novel drugs or agricultural chemicals. Future research characterizing the anti-virus mechanisms of PACs will provide further insight into the use of antiviral plant agents as medicine and agricultural chemicals.

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